

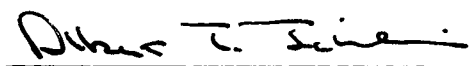
To the Graduate Council:

I am submitting herewith a thesis written by Steve Foster entitled "Natural Killer Cell Resistance in Sublines of the Hematopoietic Stem Cell Line K-562." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Microbiology.



Carl J. Wust, Major Professor

We have read this thesis
and recommend its acceptance:



Accepted for the Council:



The Graduate School

NATURAL KILLER CELL RESISTANCE IN SUBLINES OF THE
HEMATOPOIETIC STEM CELL LINE K-562

A Thesis
Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville

Steve Foster

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DEDICATION

This work is dedicated to my parents, Mr. and Mrs. William S. Foster, and to Ms. Sarah A. Edmonds for her patience and interest with respect to the day-to-day trials of research.

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I sincerely appreciate the support, patience, and interest shown by the members of my thesis committee Dr. Robert N. Moore, Dr. Albert T. Ichiki, and especially Dr. Carl J. Wust. More generally, I would like to express my appreciation for the help given me by faculty, staff, and students of the Department of Microbiology.

ABSTRACT

The pluripotent stem cell line, K-562, derived from a patient with chronic myelogenous leukemia in blastic crisis (46, 49, 50), is a commonly used target cell for studies of human natural killer (NK) cell activity. Sublines have been established from the original K-562 line, and have shown different phenotypic characteristics. These K-562 sublines were used in a study to examine possible variability in their susceptibility to NK-mediated lysis. Predominantly hypodiploid K-562 sublines, established early in passage of the parental line, were relatively resistant to lysis by NK, whereas hypotriploid lines were sensitive.

A cloned hypodiploid subline B₁V, was studied to characterize the basis for resistance to NK activity in hypodiploid K-562. Cells of this NK-resistant clone did not effectively compete with sensitive sublines in cold target inhibition assays, but were found to form conjugates with NK cells at levels similar to sensitive sublines. Furthermore this clone was susceptible to lysis by antibody-dependent cell-mediated mechanisms as well as by specific anti-K-562 serum plus complement. Together these data suggest that NK cells can recognize and bind to the resistant clone B₁V, but are not activated to mediate lysis.

The failure of B₁V targets to activate the NK cell lytic mechanism was independent of surface sialic acid. High salt extracts of B₁V did not inhibit lysis by NK cell as did those from NK-sensitive K-562 sublines. Thus, B₁V may lack surface protein determinants involved in the activation signal.

This study provides direct evidence for a target cell-associated signal for activation of the NK cell. This signal may represent an additional mode of regulation of NK activity in vivo.

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LIST OF ABBREVIATIONS

ADCC	Antibody-dependent cellular cytotoxicity
ADCMC	Antibody-dependent complement-mediated cytotoxicity
CML	Chronic myelogenous leukemia
CPM	Radioactive counts per minute
⁵¹ Cr	Radioactive chromium fifty-one
HPBL	Human peripheral blood lymphocytes
IgG	Immunoglobulin G
IL-2	Interleukin two
LAK	Lymphokine-activated killer cells
LGL	Large granular lymphocyte
NCS	Newborn calf serum
NK	Natural killer(s)
NKCF	Natural killer cytotoxic factor(s)
Ph ¹	Philadelphia chromosome
RPMI	RPMI 1640 growth medium
3M KCl	Three molar potassium chloride

CHAPTER I

INTRODUCTION

Natural killer (NK) cells represent a population of non-T, non-B lymphoid cells that have the functional capability to lyse a variety of tumor, virus-infected, and hematologic cell types (28, 32, 33). A role has been prescribed for NK cells in immunosurveillance against tumors and control of tumor metastases (31) as well as a role in modulating normal bone marrow progenitor cell proliferation (23, 24, 31).

The specific requirements for recognition and lysis of target cells by NK have not been clearly defined as of yet. It is known, however, that sensitivity to lysis by NK is directly related to the differentiative state of the target cell (82, 83). This is presumably dependent upon the presence of antigens associated with particular stages of differentiation.

The leukemic K-562 cell line is highly sensitive to NK activity (41) and has become a standard target cell for studies of human NK. The susceptibility of K-562 cells to lysis by NK appears to be related to its putative status as a pluripotent hematologic stem cell (23, 46, 50).

The studies detailed herein represent an attempt to understand the basis for the exquisite susceptibility of the K-562 cell line to NK-mediated cytotoxicity. The basic approach used was a comparison of a number of characteristics, including NK targetability, in different sublines established from the original K-562 parent line.

Cloned K-562 sublines have been shown to vary in their degree of

lysis by NK cells (43, 52). Attempts to define the basis for loss of NK susceptibility in these lines have not been conclusive (15). In addition, K-562 sublines maintained in the laboratory of Dr. Carmen B. Lozzio have exhibited marked variation in a number of characteristics. These include variations in modal chromosome number and the presence of particular chromosomal markers, as well as the differentiative capacity of K-562 sublines as defined by cell surface phenotype (50, A. T. Ichiki, manuscript in preparation).

The aim of this study was to examine sublines of K-562 which show decreased susceptibility to NK-mediated cytotoxicity and characterize the basis for their decreased reactivity, in an attempt to relate this resistance to other characteristics.

CHAPTER II

SURVEY OF THE LITERATURE

Characteristics of Natural Killer Cells

Natural killer (NK) cells have been described as populations of lymphoid cells in rats, mice, and humans with an intrinsic capability to recognize and lyse a variety of cell types. Susceptible NK targets include fresh and cultured tumor cells, virus-infected fibroblastic cells, bone marrow progenitor cells, and lymphoblastoid B-cells (6, 24, 32, 41). Lysis by NK cells does not require prior exposure to antigen or the presence of target sensitizing antibody. Contrary to the histocompatibility restriction found with cytotoxic T cells, NK cells can lyse cells of different strains and species (32, 33).

The broad, spontaneous reactivity of NK cells has been the basis for proposing their role in immunosurveillance against tumor growth and metastases, in response against infection, and in regulation of hematologic progenitor cell proliferation (23, 31, 32). The in vivo importance of NK cells in tumor surveillance is underscored by the demonstrably high incidence of spontaneous tumors in the selectively NK-deficient beige mouse strain (63) and in humans with Chediak-Higashi syndrome who also manifest inactive NK cells (64). NK cells have been shown to be involved in clearance of injected tumor cells in vivo (32).

Morphologically NK cells have been characterized as large granular lymphocytes (LGL) found in the peripheral blood, spleen, and lymphatics

(44, 59, 79). The cells do not adhere to plastic or nylon wool (79) and may be readily enriched by fractionation of peripheral blood on Percoll step gradients (76, 78). Effector cells that bind to NK-sensitive tumor targets are relatively large, with a low nuclear to cytoplasmic ratio. Characteristic azurophilic granules are seen in the cytoplasm after Giemsa staining (79). LGL comprise approximately 10% of human peripheral blood lymphocytes. It has been estimated that roughly 50% of the LGL are active NK cells (75).

NK cells do not represent either mature T or B lymphocytes. Rather they are thought of as representing a functionally specialized lymphoid class derived from T cell progenitors. NK cells express surface antigens characteristic of T cells (OKTII) and proliferate in response to interleukin-2 (IL-2) (57). Exposure of NK cells to thymic hormones has been shown to induce the expression of additional T cell antigens with a concomitant loss of NK functionality (72).

The maturation of human NK cells is thought to require both IL-2 and interferon (IFN) (60) and can be followed on the basis of surface marker expression. Two characteristic antigen markers OKMI, a monocytoïd differentiation antigen, and HNK-1 are found on mature NK cells (2). OKMI is absent on less mature NK cells but appears during functional maturation. The HNK-1 marker is present on essentially all NK cells (2). Approximately 15% of adult peripheral blood lymphocytes express HNK-1.

Specificity of Natural Killer Cells

The specificity of target cell recognition and lysis by NK cells has not yet been clearly elucidated. The range of targets is quite broad, suggesting heterogeneity amongst the NK effector cells, as well as among the structures recognized on the target. As shown by cold-target inhibition and target monolayer adsorption studies, NK cells reactive with cultured tumor cell lines are clonally distributed with respect to target specificity (29). These clonal NK subpopulations each recognize a limited number of related target determinants, the broad overall reactivity of NK cells being accounted for by a spectrum of these clonal subpopulations. For example, the NK cells responsible for lysis of herpes simplex virus infected fibroblasts are not involved in lysis of tumor targets (15).

One primary specificity exhibited by NK cells is that for hematologic cell types, including freshly isolated leukemic cells (91), cultured leukemia lines such as the K-562 cell line (41, 49), and immature cells in the bone marrow (23, 24). This reactivity with normal and abnormal hematologic cells is apparently related to an undifferentiated state in the target cell. For example, fetal bone marrow cells are lysed to a greater extent than adult bone marrow cells (24) probably because of the presence of a greater number of immature hematologic progenitors. Reactivity of NK cells with hematologic progenitors has been demonstrated directly via the inhibition by NK cells, of granulocytic colony formation in vitro (23). These observations suggest a role for NK cells in the maintenance of bone marrow homeostasis.

The K-562 cell line is highly susceptible to lysis by human NK cells (41) and classical NK activity is most often defined in terms of lysis of K-562 cells. This tumor cell line appears to represent unrestricted growth of a pluripotent hematologic stem cell (46, 50). K-562 cell susceptibility to recognition and lysis by NK cells is lost when the K-562 cells are induced to differentiate (82, 83) suggesting that NK specificities are directed to immature structures on the K-562 cell. It has also been shown that K-562 cells are able to compete for NK-mediated lysis of bone marrow cells (23). Together, these results suggest that there is homology between structures on NK-sensitive bone marrow populations and those found on K-562 cells. Cold target inhibition studies indicate that K-562 cells express a "dominant" NK recognition entity that may or may not be present on other tumor cell targets (29). This dominant entity could be related to the aforementioned stem cell homology.

The reactivity of NK cells is not confined to hematologic cell types. Lysis of cells from solid tumors has been demonstrated with human NK cells (71). The lysis of solid tumors has also been shown to involve the so-called lymphokine-activated killer cells (LAK) generated in mixed lymphocyte culture or in response to lymphokines (22). These activated killer cells are able to lyse fresh autologous tumor explants which are often resistant to classically defined, spontaneous NK activity. LAK apparently represent a distinct functional subset of LGL (22). Their exact relationship to NK cells is unclear.

Concise definition of the target antigens recognized by NK cells has proven to be an elusive task, probably owing to the broad

reactivities found in this system. The antigens recognized on the K-562 cell and many other NK target cells are believed to be protein or glycoprotein in nature since they are sensitive to mild protease treatment (35). In addition, membrane-bound glycoproteins isolated from NK-sensitive targets have been shown to be capable of inhibiting the binding of NK cells to their targets (66).

Solubilized proteins of K-562 cells, separated by polyacrylamide gel electrophoresis inhibit NK cell binding (56). Three particular protein fractions with molecular sizes of approximately 200, 120, and 80 kilodaltons, were identified as putative human NK recognition antigens. Consistent with cold target inhibition data (1), the 200 kilodalton species was found only on the K-562 cell and not on two other leukemic cell lines (56).

A correlation has been noted between expression of receptors for transferrin and the susceptibility of various leukemic cell lines to lysis by NK cells (81). In addition, an affinity purified tryptic fragment of the transferrin receptor was shown to inhibit NK activity against K-562 cells. Thus the transferrin receptor may be part of a complex antigen structure on NK-sensitive target cells (81). Transferrin receptor is present on both normal and malignant proliferating cells, therefore this receptor could represent a homologous recognition structure on normal and malignant NK targets.

The Cytolytic Process of Natural Killer Cells

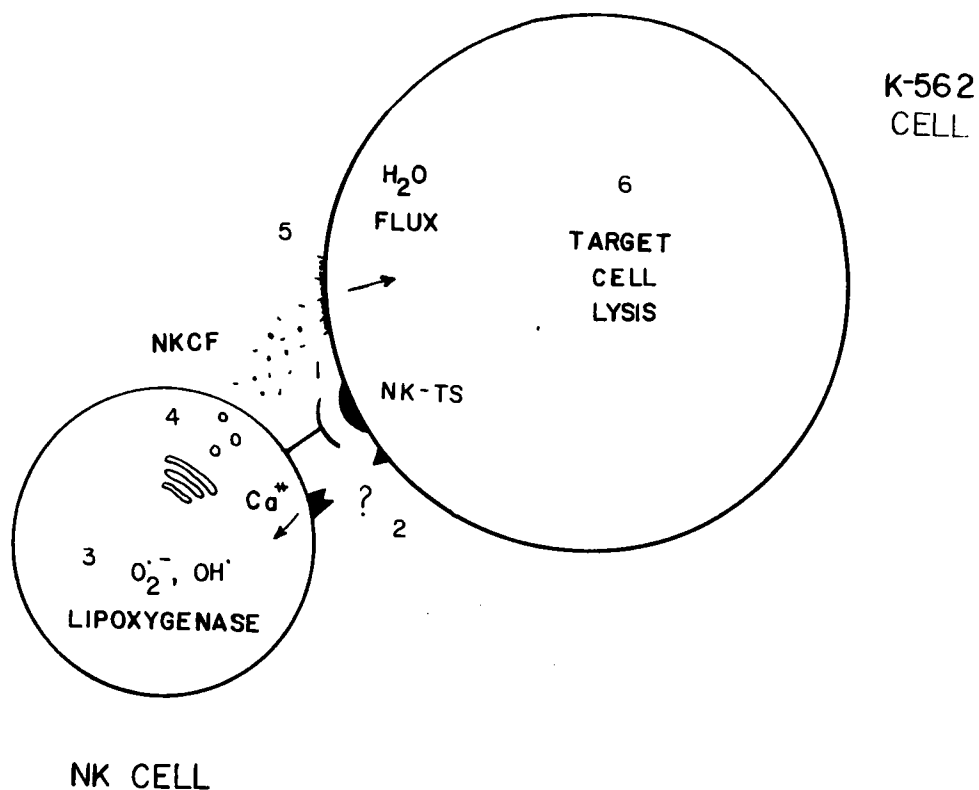
The actual cytolytic process carried out by NK cells may be divided into four functional stages as listed below:

1. NK recognition of, and binding to, the target cell;
2. activation of the NK cell lytic mechanism;
3. delivery of cytotoxic agents or "lethal hit";
4. target cell death and the recycling of the NK cell to a second target.

These stages are represented schematically in figure 1.

Target cell binding is observed as the formation of rosette-like NK/target conjugates (21). Recognition is likely dependent upon a specific NK-associated receptor although this receptor has yet to be clearly identified. Binding itself is rapid, occurring in less than one minute. It is dependent on the presence of magnesium and may occur at temperatures less than 37°C (34). Electron microscopic studies have suggested interdigitation of the NK and target cell membranes in the region of contact (8). Uropodal structures have also been described on NK cells and may be involved in the target-binding event (55).

NK cells are also capable of interacting with targets through target-bound antibody as virtually all human NK cells express Fc receptors for IgG (30). Thus, NK cells are probably involved in killer (K) cell lysis of antibody sensitized targets. Microscopically a single LGL has been shown capable of lysing both antibody-coated and non-coated target cells (5), sequentially killing one target and then the other. K cell mediated lysis appears to be more rapid than NK-mediated lysis, possibly because of a more rapid activation of the effector mechanism via the Fc receptor as opposed to activation via the putative NK receptor.



NK-MEDIATED CYTOLYSIS:

1. Recognition and binding of target cell.
2. NK-cell activation.
3. Early effector metabolism.
4. Delivery of lethal hit.
5. Target cell programming and effector recycling.
6. Target cell death.

Figure 1. Schematic representation of NK-mediated cytotoxicity.

A distinct activation step in the NK cytolytic process has been demonstrated. Mild trypsinization of LGL causes the cells to be incapable of carrying out lysis although the frequency of effector/target conjugates is unchanged (14). A monoclonal antibody reacting with the T-200 glycoprotein complex on LGL, blocks cytolysis of K-562 at this activation stage (74). Apparently mild trypsinization also affects the T200 complex (14). Thus, this protein complex may be involved in the activation events occurring subsequent to target cell binding, presumably interacting with some structure on the target cell.

One early event detectable upon triggering of the NK cell for lysis is a burst of oxidative metabolism measurable as luminol-dependent chemiluminescence (65). This burst could well be the result of a calcium flux after receptor interaction (26). Scavengers of both superoxide and hydroxyl radicals have been shown to inhibit natural killing (26, 73) suggesting that these species are important components in the oxidative burst. Oxidative species of this sort have been shown to be involved in the activation of arachidonic acid metabolism (26, 37, 70). NK cell arachidonic acid metabolism particularly via the lipooxygenase pathway, has been implicated as necessary for cytolysis to occur (36).

It has also been reported that inhibitors of neutral serine proteases block lysis by NK at a stage subsequent to binding (20, 39). Proteases of this type may be involved in the early events of cytolysis, serving to activate subsequent metabolic processes.

The early activation events of the NK cytolytic process do not necessarily lead to completion of the lytic cycle. Patients with

Chediak-Higashi syndrome exhibit non-functional NK cells (64) yet LGL from these patients give a normal oxidative burst upon interaction with NK targets (26). In addition, the oxidative burst occurs at 20°C whereas target cell lysis requires a temperature of 37°C (26).

Delivery of the lethal hit or "programming for lysis" occurs rapidly 10-30 minutes after initiation of an in vitro assay. The process requires the presence of calcium (34). Programming likely involves secretion by the effector cell since there is orientation of the golgi apparatus towards the target cell (8). Monensin, a carboxylic ionophore which blocks golgi functions, prevents lysis (68), which is consistent with the proposed involvement of secretory mechanisms.

With respect to the actual lytic moiety, soluble NK cytotoxic factors (NKCF) have been identified in the supernatant fluid of NK/target co-cultures after incubation (13, 87, 88). The generation of NKCF is induced specifically by NK susceptible targets and is considered to be the product of LGL (13). NKCF are protease sensitive and will not pass through a dialysis membrane, suggesting that they are likely proteins with a molecular weight of greater than 12,000 (30, 60). NKCF lyse only NK-sensitive target cells (13, 87). Susceptibility of the target to NKCF is dependent on the presence of a protease insensitive receptor (13, 35), which is possibly carbohydrate in nature since simple sugars and hexose phosphates have been shown to inhibit lysis by NKCF (17, 87).

Analogously to cytolysis mediated by T cell lymphotoxins, NKCF appear to operate through formation of a macromolecular complex. A

papain-sensitive moiety is apparently transferred to the target cell along with NKCF, and participates in the lytic event (35). Since NKCF are not sensitive to papain, this "lytic focusing molecule" is believed to complex with NKCF to form the active killing unit.

Granules isolated from rat LGL tumors have been shown to contain proteins capable of forming cylindrical pore-structures on target cell membranes (54). These structures mediate target cell lysis, however they exhibit a different specificity in killing than that described for NKCF. The relationship between these granule-associated lytic agents and NKCF remains to be elucidated.

After transfer of the lytic complex, the NK cell may dissociate from the target and migrate to a second target cell. Recycling of the NK cell is not immediate because a lag of approximately two hours is necessary before a new target can be bound (77). Interferon augments NK activity (12) primarily by accelerating the kinetics of NK cell recycling (77), which eliminates the lag in binding capacity.

Death of the target cell apparently represents colloid osmotic lysis (34, 35). The cell membrane is permeabilized, allowing an influx of water, and leading to lysis. This process may occur subsequent to dissociation of the effector/target conjugate (56).

The K-562 Cell Line

The K-562 cell line was established in 1970 from the pleural effusion of a patient with chronic myelogenous leukemia (CML) in terminal blastic crisis (49). Since that time, the cell line has been maintained in continuous culture and has continued to express the

Philadelphia (Ph¹) chromosome marker characteristic of CML (46, 50).

The cells can be stored in liquid nitrogen and are available at various passage intervals. Sublines of K-562 have been established from these frozen stocks.

K-562 cells are morphologically undifferentiated. The cells are typically round, 14-17 μ m in diameter, although they may vary from 30 μ m to less than 10 μ m in size. The cytoplasm is strongly basophilic and agranular, with a few small vacuoles. The nuclei are pleomorphic with round and slightly indented forms predominating. The nuclei typically contain 1-4 nucleoli. The cells are not phagocytic and do not express surface or secreted immunoglobulin. Viral antigens have not been detected and screening for mycoplasma is routinely negative (49, 50). K-562 cells grow rapidly in suspension culture with a mean generation time at 10-12 hours. This generation time became stable at passage 85. The cells can be cloned with high efficiency in soft agar (49, 50).

The Ph¹ chromosome in the K-562 cell has been characterized as a reciprocal translocation between chromosomes 22 and 9 t (22; 9) (q11; p23). Other chromosomal abnormalities have been noted including an additional translocation, t (15; 17) (q21; q24), chromosomal ring structures, and deleted portions. These abnormal structures vary among K-562 sublines suggesting chromosomal clonal evolution both in the patient of origin and in the cultured cells themselves (49, 50). Sublines established from early passages usually show a near diploid modal chromosome number whereas sublines established later (beginning at passage number 69) tend to show a near triploid karyotype (50).

A finding which could have significance is the mapping of the human c-abl proto-oncogene within the region of chromosome 9 that is translocated in the Ph¹ chromosome (3, 25). This finding, coupled with the detection of c-abl translation product in K-562 cells (O. Witte, personal communication), suggests a possible role for c-abl in the production or maintenance of the leukemic state. Studies on induced differentiation of the myeloid leukemia cell line, HL-60, have indicated that the c-abl gene may be involved in normal myeloid cell function at various stages of differentiation (85). Thus, a gene that appears to be involved in normal hematopoiesis may contribute to the leukemic state.

Considerable evidence has accumulated to support the contention that K-562 cells represent a cancerous homolog of the pluripotent hematopoietic stem cell (reviewed in 50). CML has been considered to be a clonal tumor arising at the pluripotent stem cell level (16). However, at blastic crisis, patients usually present with proliferating cell populations phenotypically representing either the myeloid or lymphoid differentiative lineages (16). The K-562 line appears to be unique in that the cultured cells express markers of both lineages (46, 50).

As indicated by the expression of cell surface markers, K-562 cells are apparently capable of partial differentiation along all five of the major bone marrow cell lineages. For example, the granulocytic marker D5, the B-lymphoid antigen BA-1, and the T-cell antigen OKT17, have all been found on K-562 cells (50, A. T. Ichiki in preparation). Platelet glycoproteins and fetal hemoglobin have been reported as well

(18, 61). Enzyme markers characteristic of the separate lineages have also been noted (46, 49, 50).

Partial differentiation of K-562 cells occurs spontaneously with age in culture or, more rapidly, in the presence of various chemical inducers. Induction of erythroid differentiation and hemoglobin production by hemin (18) is probably the most cited example of induced differentiation in K-562. The differentiation of K-562 cells appears to be readily reversible when the cells are passaged in fresh medium. Terminal differentiation to mature myeloid cell types, as is observed with chemical induction of HL-60 cells, has not been demonstrated with K-562 cells (11, 50).

Of particular interest with K-562 cells has been the characterization of its immunological properties as related to its differentiative state and/or tumor-associated components. Such properties are important both with respect to practical application, which could lead to effective immunotherapy of leukemia, and to an understanding of tumor antigenicity.

Much of the immunologic characterization of the K-562 line has involved the use of xenogeneic anti-K-562 sera produced in goats. Such antisera is extensively adsorbed with normal human peripheral blood leukocytes and/or bone marrow cells. The sera then exhibit leukemia-specific cytotoxicity mediated by complement or cellular effector mechanisms (47, 86). Whole antiserum, gamma globulin, or Fab fragment is also able to cause death of K-562 cells in the absence of complement or effector cells in vitro (51, 89), and in de complemented mice in vivo (48).

A leukemia-specific protein antigen has been described on K-562 cells (10). The protein was isolated and found to inhibit the specific complement-mediated cytolysis of goat anti-K-562 antibody. It was found to be nonglycosylated and have an apparent molecular weight of 80,000.

Other studies showed that adsorbed goat antiserum inhibited the formation of rosettes at the Fc receptor between K-562 cells and hemolysis-sensitized sheep erythrocytes (40). This inhibition suggested that the leukemia-associated antigen on K-562 cells could be associated with an Fc receptor, which was antigenically different from that on mature leukocytes. However, anti-K-562 antibody reacts with non-Fc bearing leukemic cells suggesting the presence of additional determinants (58).

Anti-K-562 monoclonal antibodies have been shown to recognize bone marrow progenitor cells. Normal bone marrow was treated with these antibodies plus complement, and both myeloid and erythroid colony formation in soft agar was inhibited (90). Another monoclonal, produced against normal bone marrow, reacts strongly with K-562 cells yet recognizes less than three percent of normal bone marrow cells (69). This monoclonal (E10) is capable of lysing K-562 in the absence of effector cells or complement (Ichiki, personal communication). These results suggest direct antigenic homology between K-562 cells and bone marrow progenitors.

Possibly the most widely studied immunological feature of K-562 cells is their susceptibility to human NK-mediated cytotoxicity, which was first demonstrated in 1976 (41). Since that time, K-562 cells have

become established worldwide as the standard target cell for studies of human NK activity. As noted earlier, the NK sensitivity of K-562 cells appears related to their undifferentiated state and at least some degree of homology with bone marrow stem cells (23, 82, 83). This relationship promotes the continued study of K-562 cells and provides the basis for the studies described in this thesis.

CHAPTER III

MATERIALS AND METHODS

Cell Culture

K-562 cells were kindly provided by Dr. Carmen B. Lozzio. The cells were cultured in RPMI 1640 (Gibco Lab., Grand Island, NY) supplemented with 10% newborn calf serum (NCS) (Gibco) and 5 μ g/ml of gentamycin (Gibco). A number of K-562 sublines were obtained from stocks frozen in liquid nitrogen. All K-562 sublines were grown as suspension cultures in tissue culture flasks (Nuncalox 80 cm², Gibco).

The murine cell line L929 was also used. These cells were grown as monolayer cultures in 80 cm² flasks (Gibco). The medium used was McCoy's 5A (Gibco) supplemented with 10% NCS and 5 μ g/ml of gentamycin.

Isolation of Human Peripheral Blood Lymphocytes

Human peripheral blood lymphocytes (HPBL) were obtained by fractionation of whole venous blood from healthy donors. Heparinized blood was diluted two-fold with RPMI and 10 ml was layered on 5 ml of Histopaque 1077 (Sigma Chem. Co., St. Louis, MO) in a 15 ml conical centrifuge tube (Corning, Fisher Scientific, Norcross, GA). The tube was spun for 35 minutes at 400 x g. Cells from the interface were harvested and washed three times in medium.

Adherent cells were removed by allowing the cells to incubate for 30 minutes at room temperature in a 175 cm² tissue culture flask (Nuncalgon, Gibco). Non-adherent cells were harvested with a pipette. This was repeated three times to insure adherent cell removal. The non-adherent cells were then placed in tubes with scrubbed nylon wool (Fenwal Lab., Deerfield, IL) at room temperature for 15 minutes. Cells not adhering were removed with a pipette, counted using trypan blue exclusion to judge viability, and suspended in RPMI, 10% NCS, at appropriate concentrations for assay of natural killer activity.

When desired, HPBL were activated with with human fibroblastic interferon (Calbiochem-Behring, La Jolla, CA). The cells at 5×10^6 /ml were incubated with 10^3 units/ml of interferon for 1 hour at 37°C and washed.

Goat Anti-K-562 Serum

Goat anti-K-562 was provided by Dr. Carmen B. Lozzio. Goats were injected at weekly intervals with whole K-562 cells in Freund's adjuvant (Sigma). For the first injection complete Freund's was used. Subsequent injections used incomplete Freund's. A final booster was given intravenously. The goats were bled and the serum obtained was heat inactivated and adsorbed five times with whole human buffy coat (Plasma Alliance). The sera were stored at -70°C.

Rabbit Anti-K-562 Serum

Female New Zealand white rabbits were given weekly subcutaneous injections of 4×10^6 K-562 cells in RPMI and lipovant (Accurate

Chem., Westbury, NY). After five injections the rabbits were bled and the serum frozen at -70°C .

Chromium Labeling of Cells

K-562 cells were washed once and suspended at approximately 4×10^6 cells in 1 ml of RPMI, 10% NCS. $\text{Na}_2^{51}\text{CrO}_4$ (ICN, Irvine, CA) was diluted 1:10 in RPMI and 400 μCi was added to the cells. After incubation for 1 hour at 37°C on a rocker panel, the cells were washed five times in RPMI and counted. The cells were suspended in RPMI with 10% NCS at 2×10^5 cells/ml and used in assays for lysis by antibody and complement or cellular effector mechanisms.

L929 cells were labeled as described for K-562 cells except 100 μCi of $\text{Na}_2^{51}\text{CrO}_4$ were used.

Chromium-Release Assays for Cytotoxicity

Ten thousand chromium-labeled K-562 cells in 50 μl were added to individual wells of a 96 well microtiter plate (Dynatech, Alexandria, VA). Cytotoxic effectors, either HPBL, antibody plus complement, or antibody plus HPBL, were added in 100 μl volumes. Spontaneous release wells contained 50 μl of labelled K-562 cells and 100 μl of RPMI. Total release wells contained 50 μl of labelled K-562 and 100 μl of 3% Triton X-100 (Sigma). After appropriate times of incubation at 37°C , the plates were centrifuged and 50 μl of supernatant fluid was harvested from each well. Radioactivity for each sample was determined in an LKB-Wallac Riagamma 1274 spectrometer. All tests were performed

in triplicate. Percent cytolysis was calculated using the following formula:

$$\% \text{ cytolysis} = \frac{\text{test well cpm} - \text{avg. spontaneous cpm}}{\text{avg. total release cpm} - \text{avg. spontaneous cpm}} \times 100$$

Results are presented as the mean percent cytotoxicity plus or minus one standard deviation.

For assay of NK activity against K-562, HPBL were isolated as described and 100 μ l added to 50 μ l of labelled K-562. Plates were incubated for four hours except where noted as otherwise. Inhibition of NK activity was assayed using 50 μ l of HPBL, 50 μ l of labelled K-562, and 50 μ l of added inhibitor. Inhibitors were either unlabelled competitor cells (cold-target inhibition assay) or soluble antigen. Control wells containing no inhibitor were assayed for comparison.

For assay of antibody-dependent cellular cytotoxicity (ADCC), 50 μ l of HPBL were added to wells containing 50 μ l of labelled K-562 and 50 μ l of appropriately diluted rabbit anti-K-562 serum. Incubation was for 90 minutes. Control cytotoxicity in wells containing no antibody was subtracted to yield specific cytotoxicity by ADCC mechanisms.

Assay of antibody-dependent complement-mediated cytotoxicity (ADCMC) involved addition of two-fold dilutions of goat anti-K-562 serum in 50 μ l to wells containing 50 μ l of labeled K-562. Rabbit complement (Low tox H, Accurate Chem., Westbury, NY) was diluted 1:8 in RPMI and 50 μ l was added. The plates were incubated for 90 minutes.

Assay for Binding of NK Cells to K-562: Conjugate Formation Assay

The conjugate formation assay was modified from the procedure described by Grimm and Bonavida (81). Briefly, K-562 cells were washed, counted, and suspended at 1×10^6 cells/ml in RPMI. HPBL were suspended at 5×10^6 cells/ml. Both cell populations were equilibrated at 30°C for 20 minutes prior to assay. 50 μl of K-562 cells and 200 μl of HPBL were added to polystyrene microcentrifuge tubes (Fisher) which were then incubated for 5 minutes at 30°C . The tubes were centrifuged at $400 \times g$ for 5 minutes. The pellet was resuspended by gently aspirating three times with a 50 μl micropipettor. The cells were loaded in a hemocytometer and the percent of K-562 with attached lymphocytes determined as follows:

$$\begin{array}{l} \text{\% conjugate} \\ \text{forming} \\ \text{K-562} \end{array} = \frac{\text{\# K-562 with attached HPBL}}{\text{total \# K-562}} \times 100$$

Two hundred K-562 cells were counted for each tube. For some experiments washed target cells were treated with 0.1% trypsin (Gibco) for 1 hour at 37°C . The cells were washed twice in RPMI with 10% NCS and assayed for binding as above.

Preparation of KCl Antigen Extracts of K-562 Cells

K-562 cells were washed twice in serum-free RPMI and 1×10^7 cells were suspended in 1 ml of 3M KCl buffered at pH 7.4 with 0.05 sodium phosphate. After 18 hours at 4°C the cells were pelleted at $20,000 \times g$ and the supernatant dialyzed against RPMI. Aggregated protein was removed before assay by centrifugation at $5000 \times g$ for 5 minutes. Protein concentration was determined using the Bio-Rad microassay for protein (Bio-Rad, Richmond, CA).

Determination of Chemiluminescent Response of NK Cells Upon Exposure to K-562 Cells

The generation of oxidative species by NK cells was assayed by measurement of luminol-dependent chemiluminescence upon NK/target interaction. HPBL were suspended at 1×10^6 cells in 0.9 ml of RPMI containing 10% NCS that had been saturated with luminol (Eastman Org. Chem., Rochester, NY), in plastic scintillation vials (Wheaton Scientific, Wheaton, NJ). The vials were then dark adapted for 30 minutes at room temperature. 1×10^7 K-562 cells 1×10^7 in 0.1 ml, were added and the chemiluminescent response measured in a Beckman LS-730 scintillation counter (Beckman, Fullerton, CA) set in the out-of-coincidence mode. Gain was set at 80%. The window setting was wide open.

Generation and Assay of Natural Killer Cytotoxic Factors (NKCF)

NKCF were generated by co-culture of interferon-activated HPBL with K-562 cells. K-562 (10^4) and 10^6 HPBL were co-cultured in 96 well, round-bottom tissue culture plates (Corning, Fisher) in a volume of 200 μ l per well. After 20 hours at 37°C the plates were centrifuged and the supernatants harvested. The supernatants were pooled, filtered, and frozen at -40°C.

NKCF were assayed for lysis of chromium-labeled L929 cells. 100 μ l of diluted NKCF was added to wells of a 96 well flat bottom tissue culture plate (Corning, Fisher) along with 30 μ l of L929 cells. The plates were incubated for 18 hours at 37°C and supernatant fluids

harvested, as described earlier, for determination of cytotoxicity by NKCF.

Karyotypic Analysis

Analysis of K-562 karyotypes was performed in the laboratory of Dr. Carmen B. Lozzio at the University of Tennessee Memorial Research Center.

CHAPTER IV

RESULTS

Susceptibility of K-562 Sublines to Lysis by Human Natural Killer Cells

The degree of lysis of K-562 sublines by human NK cells was tested. Eight sublines established at different points in passage of the parental K-562 line (Table I), were compared for lysis by NK in a standard four hour ^{51}Cr -release assay. The results indicate that there is considerable variability amongst K-562 sublines in their susceptibility to NK (figure 2). A correlation was noted between the amount of lysis and the modal chromosome number of the sublines. K-562 sublines characterized as having predominantly hypodiploid modal chromosome numbers are less sensitive to lysis by NK than are sublines where the karyotype typically shows a hypotriploid chromosome number (Table I, figure 2). This apparent correlation between modal chromosome number and lysis by NK has been seen consistently in additional assays (data not shown).

NK-mediated Cytotoxicity Against a Cloned Hypodiploid K-562 Line

Within K-562 cultures, considerable heterogeneity exists with respect to karyotype. In order to further study the relationship between modal chromosome number in K-562 sublines, and lysis by NK, a cloned hypodiploid K-562 line was obtained from the laboratory of Dr. Carmen B. Lozzio. In this clone, designated B₁V, 93% of the cells have between 41 and 45 chromosomes. Thus clone B₁V represents an

Table I. Characteristics of K-562 Sublines^a

Subline Designation	Established at Passage #	Assayed at Passage #	Karyotype Designation	Modal Chromosome Number
A	6	92	Hypodiploid	-
B	11	111	Hypodiploid	-
B ₁	11	53	Hypodiploid	-
B ₁ V	25	45	Hypodiploid	41-45 (93%)
C	62	694	Hypotriploid	-
D	93	182	Hypotriploid	-
F ₁	206	380	Hypotriploid	-
F ₂	206	220	Hypotriploid	69

^aCourtesy Dr. C. B. Lozzio.

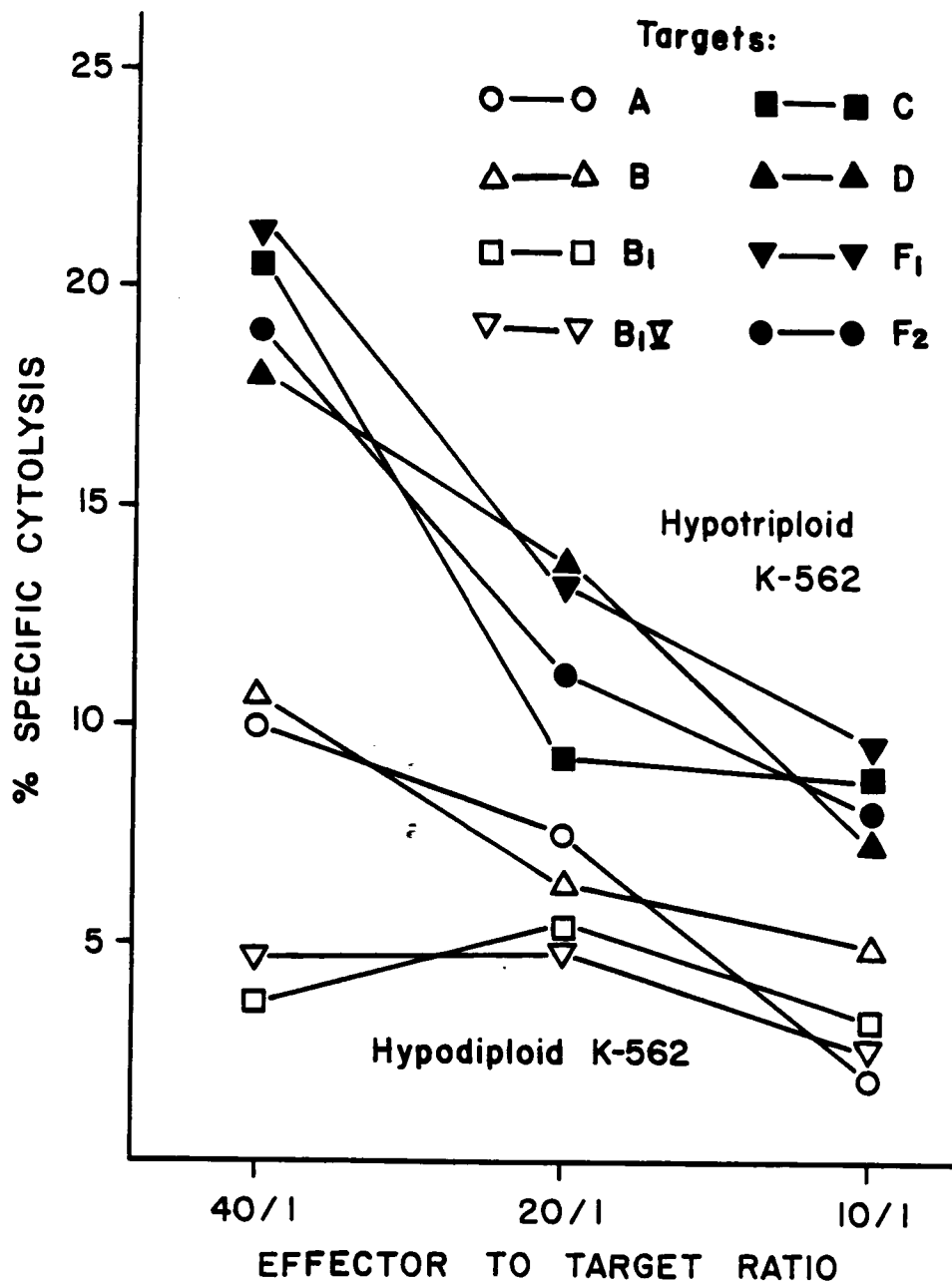


Figure 2. Lysis of eight K-562 cell sublines by human NK cells.

almost homogeneous population of hypodiploid K-562 cells, and it was used for the remainder of the studies. K-562 subline F₂ was used for comparison as a typical hypotriploid K-562 line. This subline has a modal chromosome number of 69.

F₂ and B₁V were tested for lysis by NK in a standard four hour assay. As seen in figure 3 the cloned hypodiploid line B₁V is poorly lysed by NK relative to the more typical lysis seen with subline F₂. A time course assay for NK-mediated lysis shows steadily increasing lysis of subline F₂ up to 16 hours after the start of the assay (figure 4). The small degree of lysis of subline B₁V on the other hand, appears to occur early, within the first two hours, with no additional lysis after this time. B₁V was also relatively resistant to lysis in a four hour assay with interferon-activated NK cells (figure 5).

Lysis of B₁V and F₂ by ADCMC and ADCC

The basis for resistance to lysis by NK in the hypodiploid line B₁V was examined. Assays were performed to compare the relative degrees of lysis of B₁V and F₂ by immune mechanisms other than NK-mediated lysis. Subline B₁V is lysed by both ADCMC and ADCC mechanisms (figures 6, 7); the extent of lysis was equal in both cases to that seen with the NK-susceptible line F₂. This result indicates that B₁V is susceptible to both the lytic components of complement and the lytic activity of NK/K cells activated via target-bound antibody.

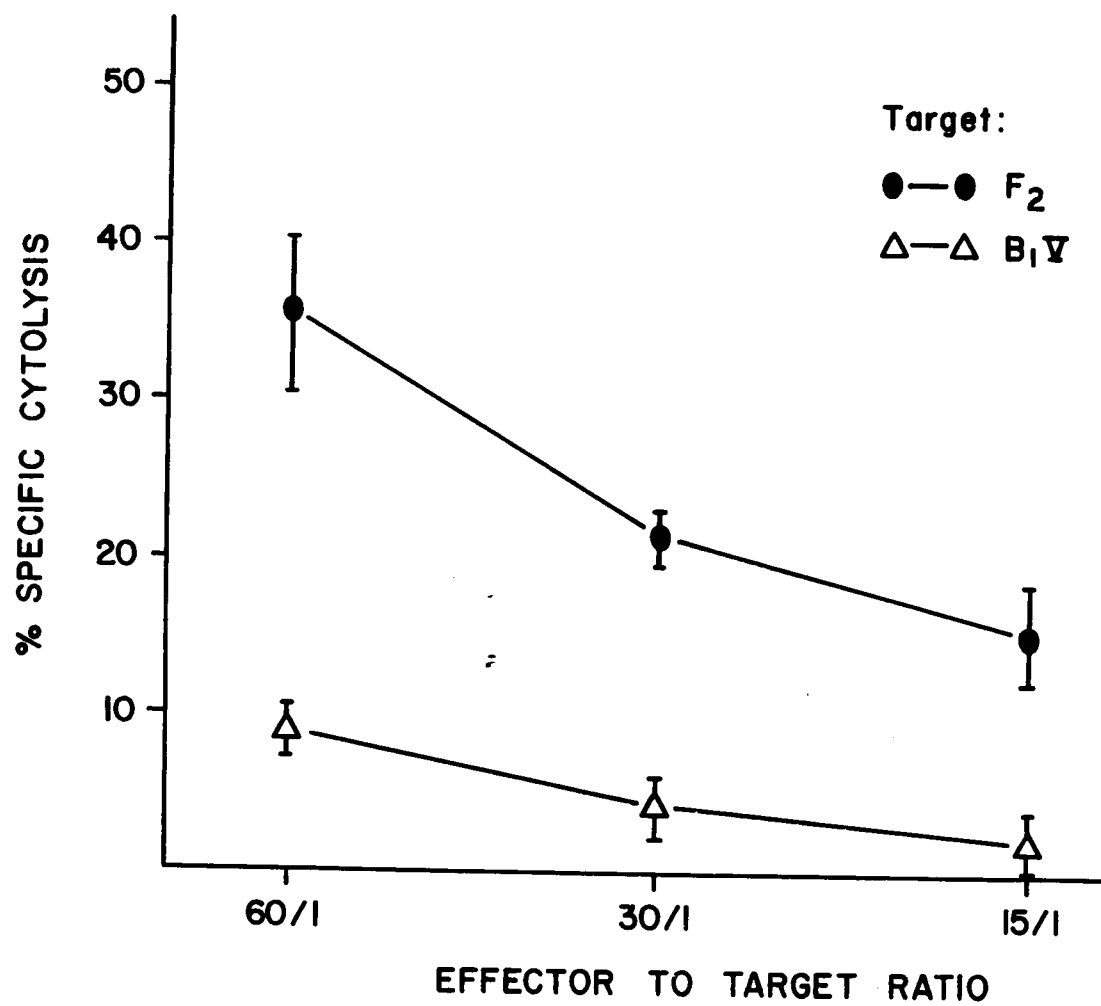


Figure 3. NK-mediated lysis of K-562 sublines B_{1V} and F_2 .

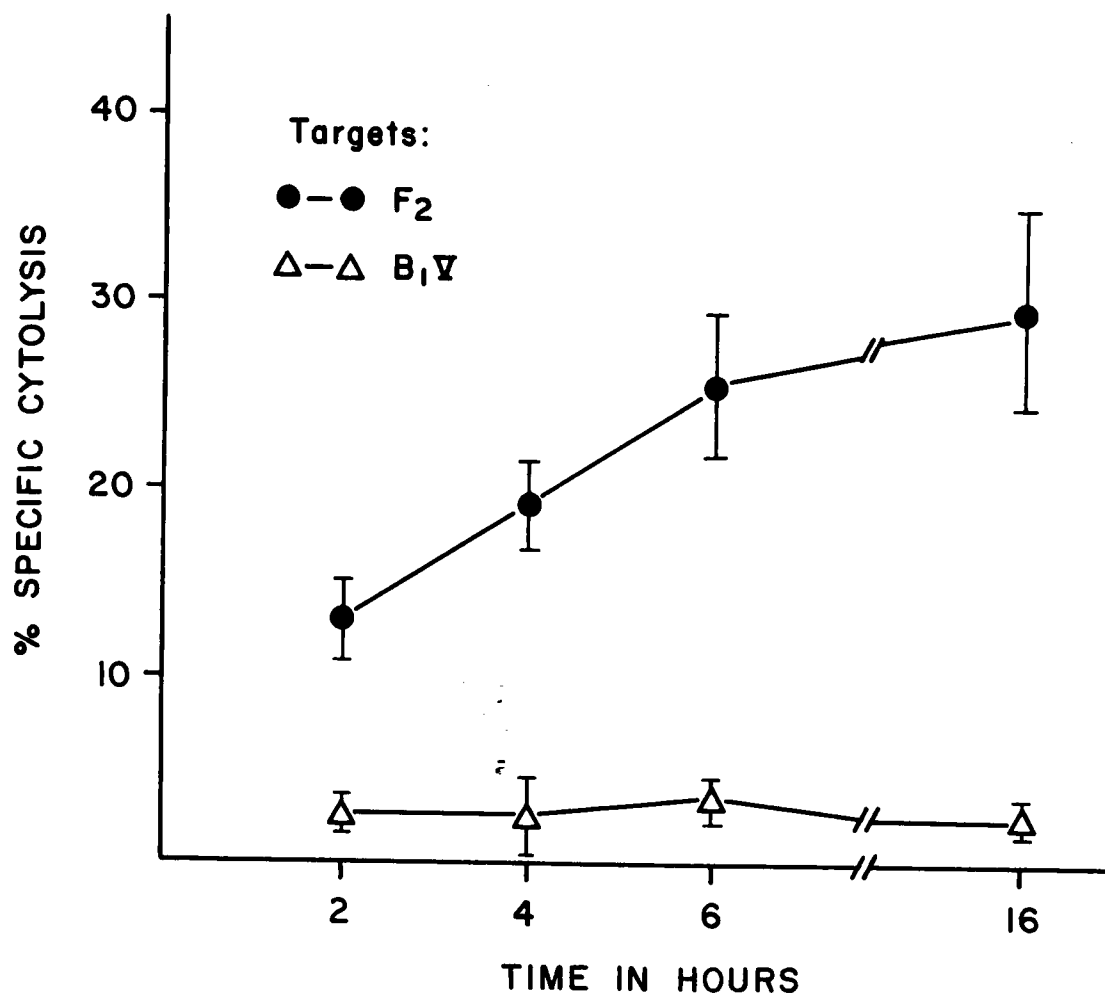


Figure 4. Time course of NK-mediated lysis of K-562 sublines B₁V and F₂. Effector to target ratio is 40:1.

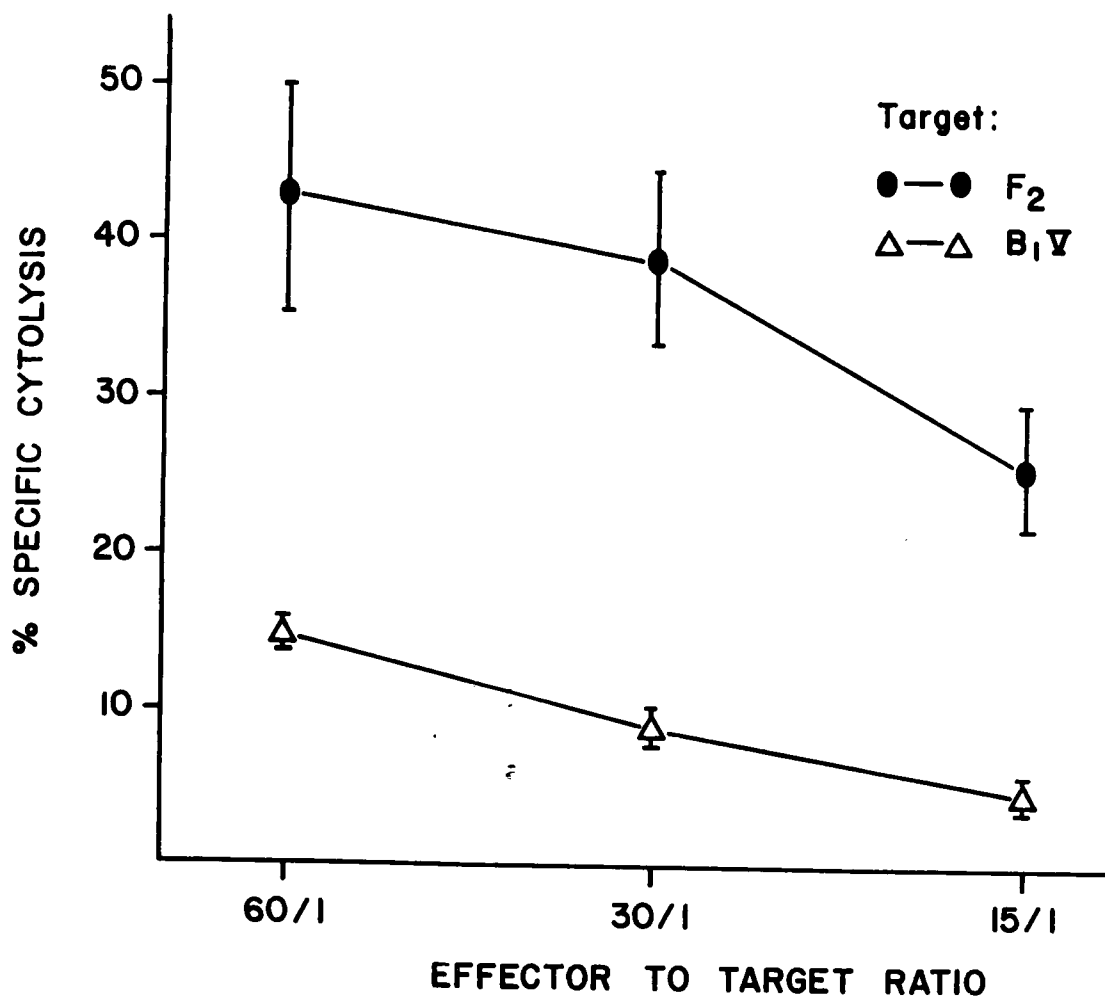


Figure 5. Lysis of K-562 sublines B₁V and F₂ by interferon-activated NK cells. HPBL were incubated with 1000 U/ml of human fibroblastic interferon for one hour, washed, and assayed for lytic activity.

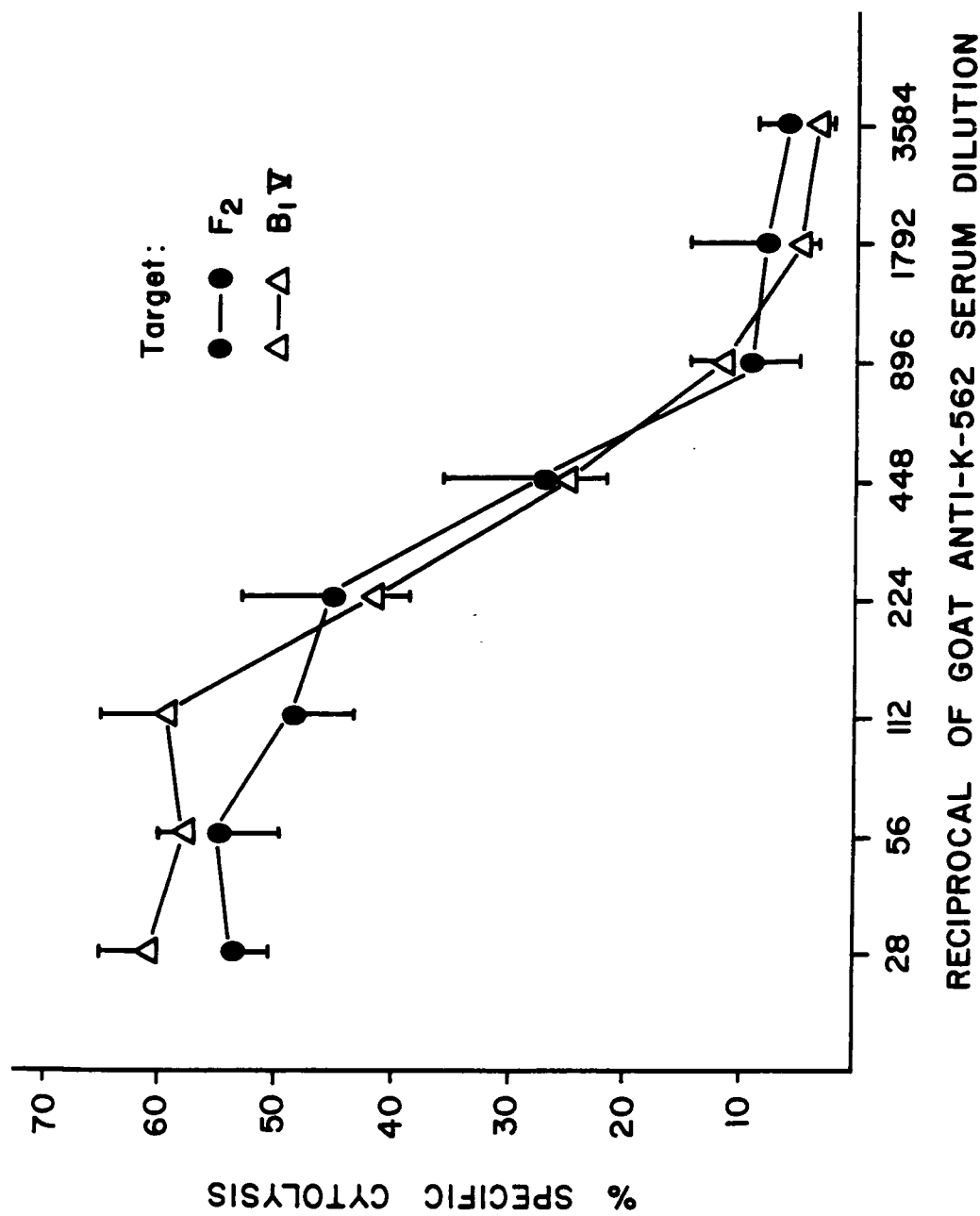


Figure 6. Lysis of K-562 sublines B₁V and F₂ by antibody and complement.

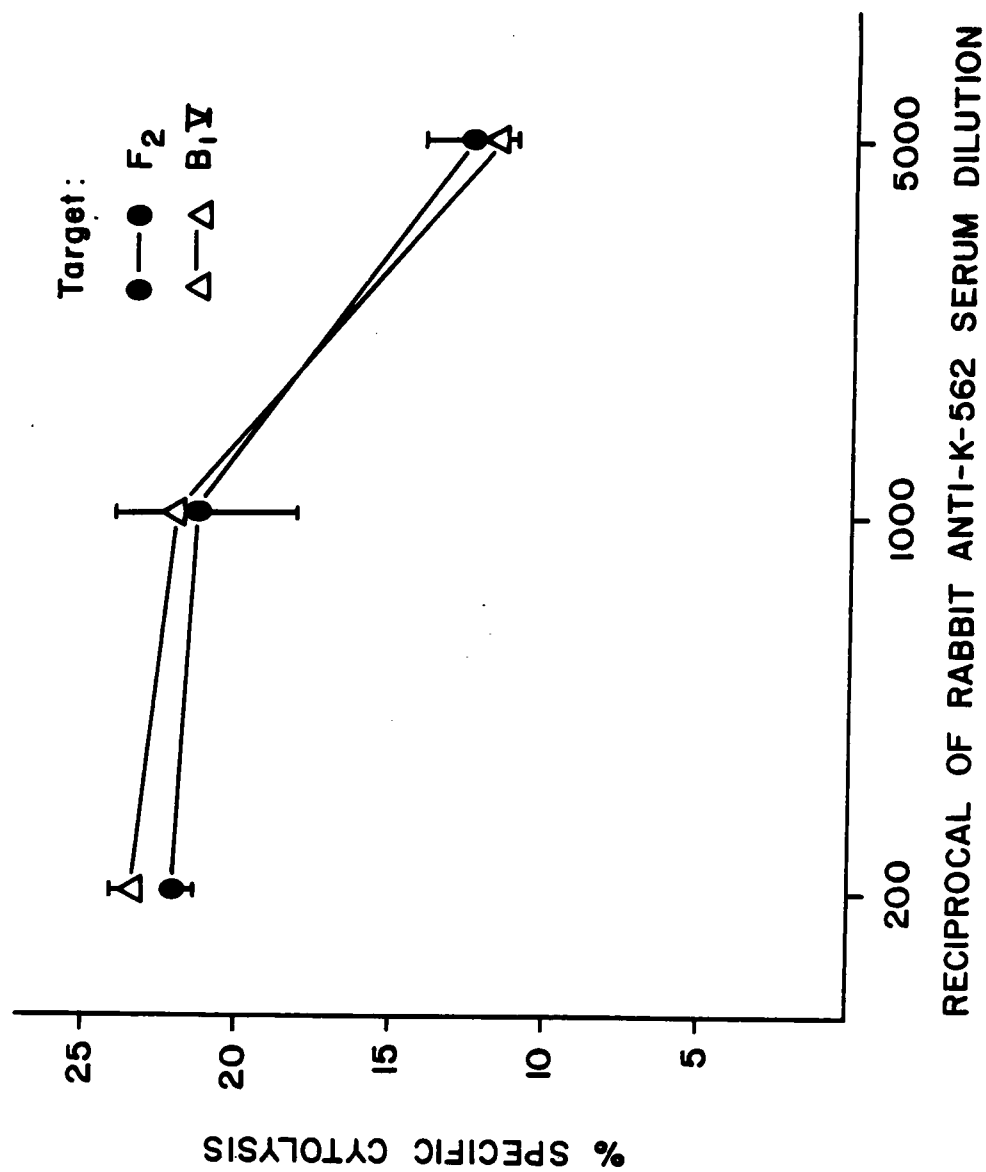


Figure 7. Antibody-dependent cellular killing of K-562 sublines B₁V and F₂. Effector to target ratio is 20:1.

Recognition of B₁V and F₂ by NK Cells:
Cold-Target Inhibition Assays and Conjugate Formation

Since the NK-resistant clone B₁V is lysed by both antibody and complement and antibody-activated cellular mechanisms, the resistance of B₁V to NK-mediated lysis would seem to lie at some stage of the NK lytic cycle other than the actual lytic event.

To test whether B₁V is recognized normally by NK cells, cold-target inhibition studies were performed to examine the ability of unlabeled B₁V and F₂ cells to compete for the lysis of chromium-labeled F₂ targets by NK cells. As observed in two separate experiments (figures 8, 9), the NK-resistant clone B₁V is consistently less able to compete for lysis than is the NK-sensitive line F₂. This is especially evident at low competitor-to-target ratios. This result would suggest that NK cells do not efficiently recognize and bind to B₁V.

However, this interpretation is disputed since conjugate-formation assays using excess HPBL show no difference in the number of B₁V targets with bound NK cells as opposed to subline F₂ (Table II). Results with another NK resistant K-562 subline, F₁, and with trypsin-treated targets suggest that the conjugate assay system is able to detect differences in the binding capacity of NK targets (Table II). These targets bind NK cells poorly.

Activation of NK Cells by B₁V and F₂: Chemiluminescent
Response and Generation of NKCF

In that NK cells appear to bind normally to the resistant clone B₁V as shown by conjugate formation, it was important to test if

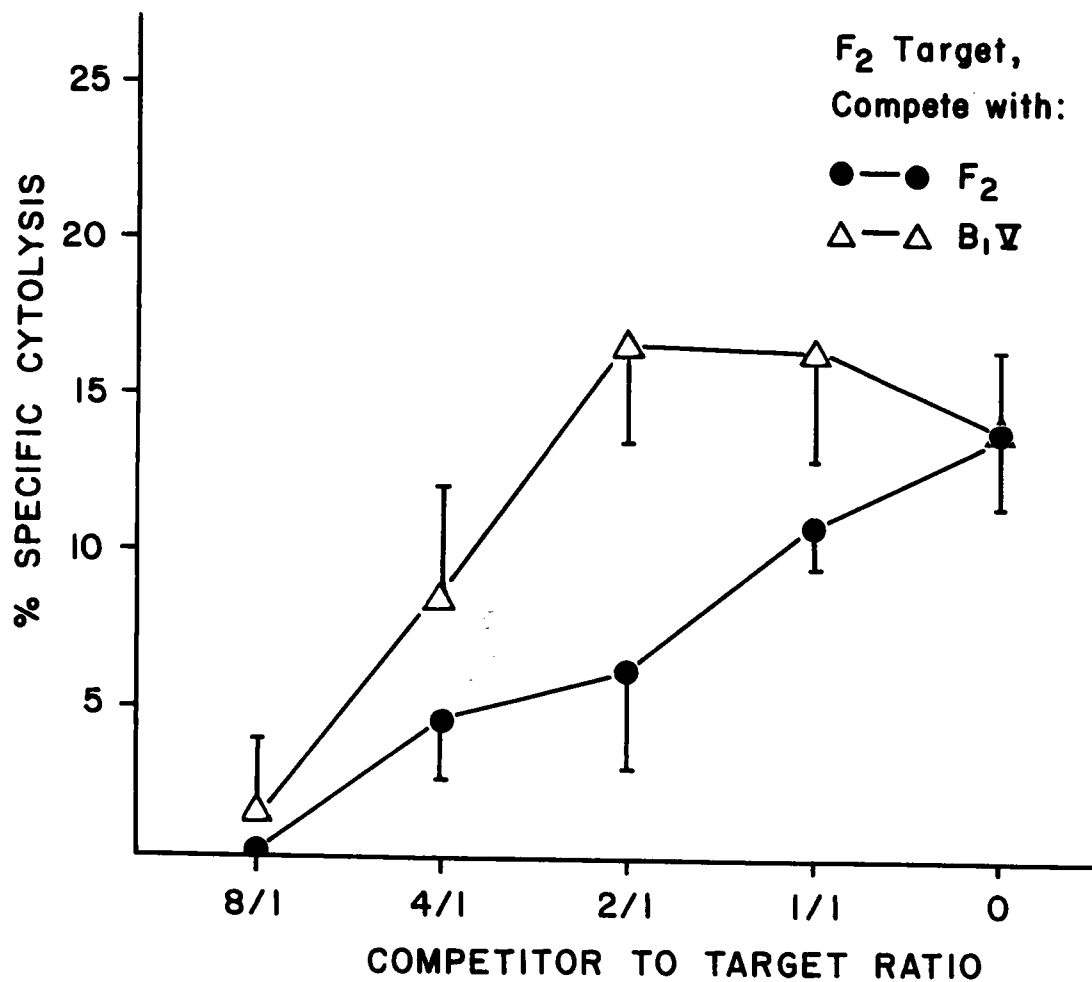


Figure 8. Cold-target inhibition of NK-mediated lysis by K-562 sublines B₁V and F₂. Effector to target ratio is 20:1.

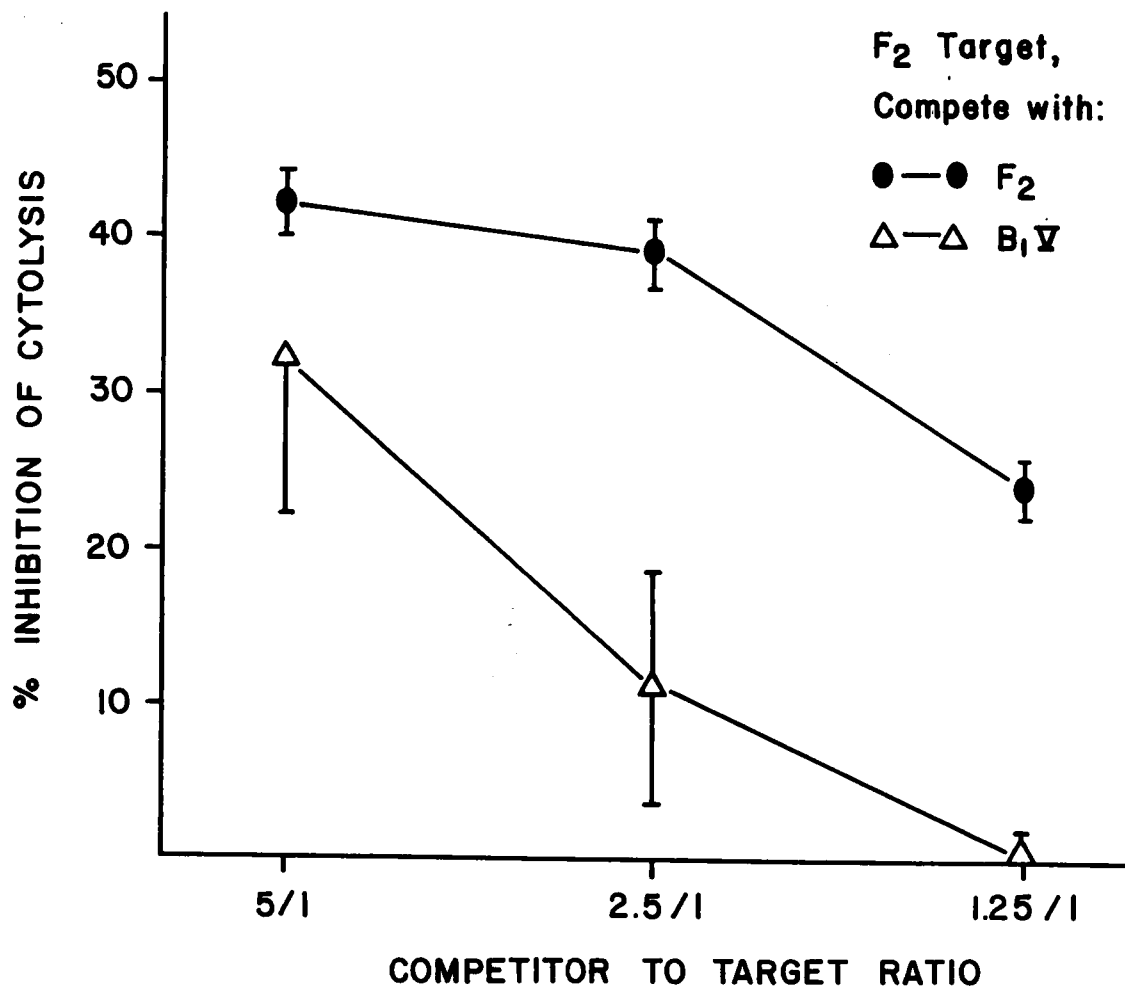


Figure 9. Cold-target competitive ability of K-562 sublines B₁V and F₂. Effector to target ratio is 20:1.

Table II. Effector/Target Conjugate Formation by K-562 Sublines

Experiment	<u>1</u>	<u>2</u>	<u>3</u>	<u>4</u>
<u>K-562 target</u>				
F ₂	50.4ab	61.4	58.4	53.0
B ₁ V	43.6	65.7	-	60.2
F ₁	-	-	23.8	-
F ₂ (Trypsin) ^c	-	-	-	32.0
B ₁ V (Trypsin)	-	-	-	25.2

^aPercent conjugate forming K-562.

^bMean of 200 targets counted.

^cK-562 targets were treated with 0.1% trypsin for one hour at 37°C, washed, and assayed for conjugate formation.

B₁V cells were able to normally activate bound NK cells. This was tested by comparing the chemiluminescent response of NK cells on exposure to B₁V and F₂. The observed chemiluminescence is indicative of the generation of oxidative species by the NK cell and presumably of its activation. As can be seen in figure 10 that the rapid spike of luminescence after exposure of HPBL to subline F₂ is not present when HPBL are exposed to B₁V. Some luminescence is seen with B₁V, but it appears to be delayed and present at relatively low levels. This result would imply that B₁V fails to normally activate bound NK cells.

Completion of the NK lytic cycle leads to the release of soluble natural killer cytotoxic factors (NKCF). To determine if the resistant clone B₁V is able to trigger the release of NKCF, HPBL were co-cultured with either B₁V or the NK-sensitive subline F₂, and the supernatant fluids tested for lytic activity against L929 cells. The results presented in figure 11 show that while subline F₂ elicits measurable NKCF activity, the resistant clone B₁V is unable to do so.

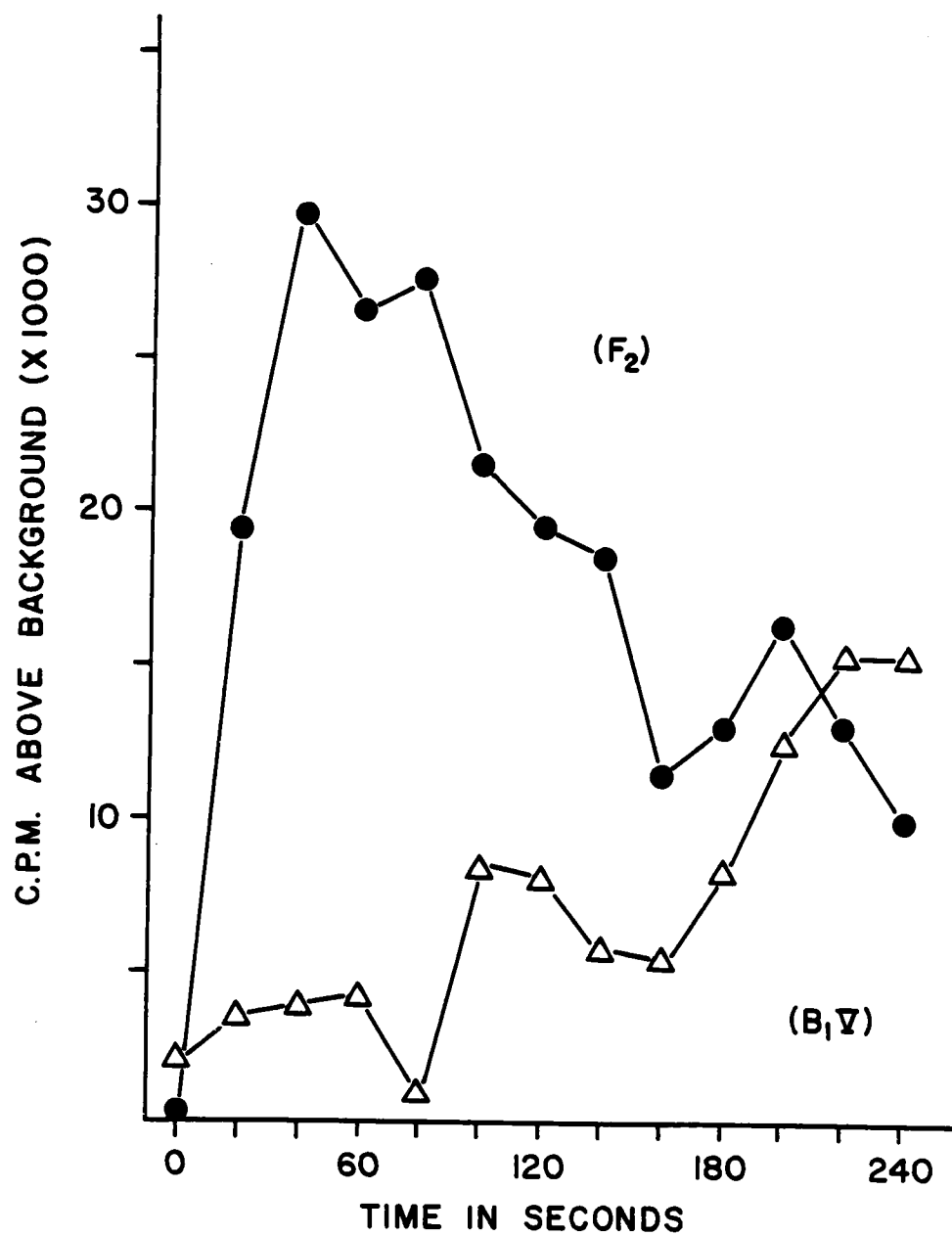


Figure 10. Chemiluminescent response of human NK cells to K-562 sublines B₁V and F₂.

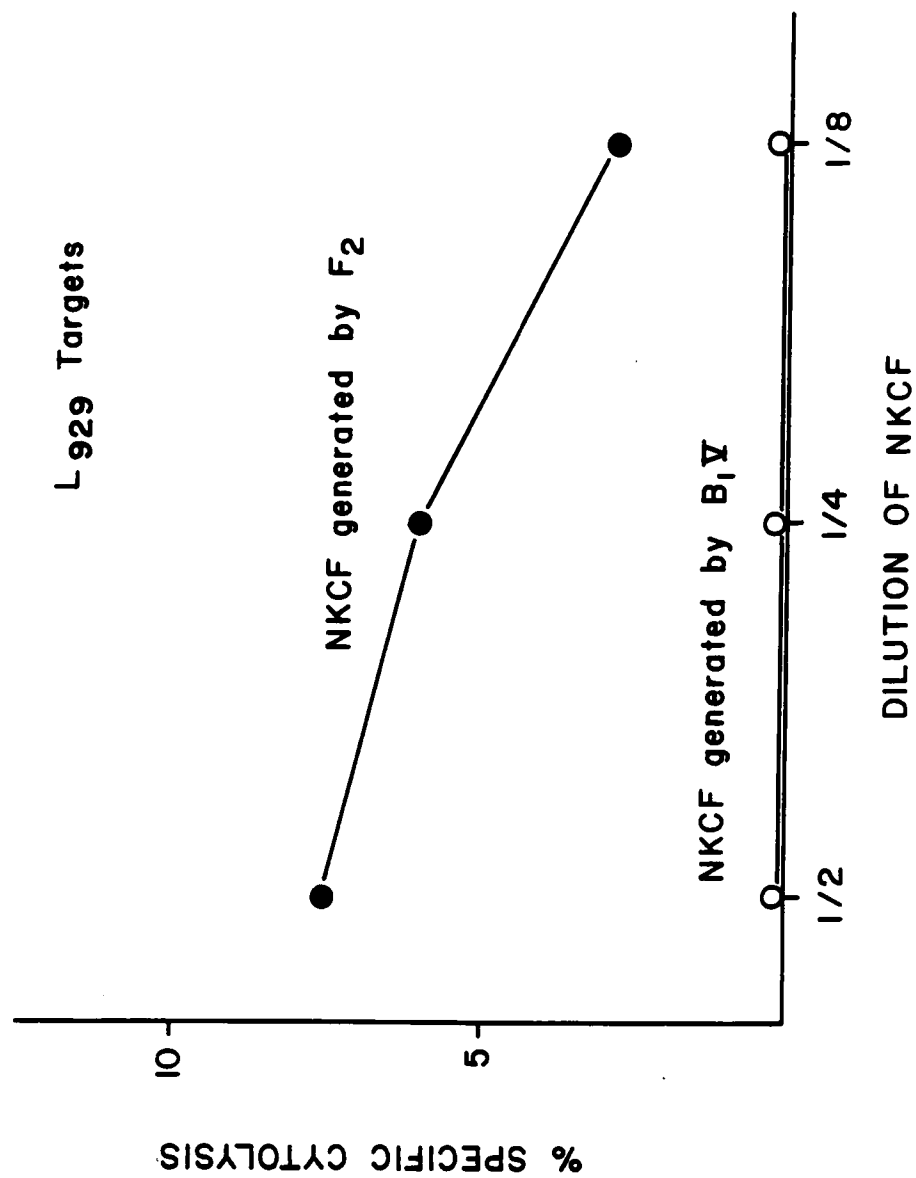


Figure 11. Induction of soluble NKCF production by K-562 sublines B₁V and F₂.

Neuraminidase Treatment of B₁V and F₂: Effect on NK Susceptibility

The relation of the resistance of clone B₁V to levels of neuraminidase-sensitive surface sialic acid was tested. B₁V and F₂ were treated with neuraminidase at 1 U/ml and tested for susceptibility to lysis by NK. Neuraminidase treatment doubled the amount of lysis seen with subline F₂ whereas lysis of B₁V was largely unaffected (Table III). The results suggest that the resistance of B₁V to NK is not related to excess levels of surface sialic acid.

Inhibition of NK-Mediated Lysis by 3M KCl Extracts of B₁V and F₂

Protein extracts of K-562 cells were prepared with 3M KCl and tested for their ability to inhibit cytolysis by NK. Extracts from subline F₂, added in a four hour NK assay against F₂ targets gave a dose-dependent inhibition of lysis (figure 12). The inhibition appears to be specific as lysis by ADCC was unaffected (figure 13). In addition, when HPBL were incubated with soluble antigen and washed before assay, no inhibition of cytolysis was seen (figure 14). Extracted antigen was unable to inhibit the binding of HPBL to K-562 in a conjugate formation assay (figure 15).

To compare extractable proteins from F₂ and B₁V for their ability to inhibit NK-mediated lysis, extracts were made from each and added in equal microgram amounts to NK assays. Extracts from the resistant clone B₁V were less able to inhibit lysis of F₂ targets than were extracts from F₂ (figure 16).

Table III. Effect of Neuraminidase (NA) Treatment on NK-mediated Lysis of K-562 Sublines

Treatment of Target:	None	1 unit NA/10 ⁶ cells
K-562 Target		
F ₂	17.5 $\pm$ 0.6 ^a	34.2 $\pm$ 2.7
B ₁ V	7.2 $\pm$ 0.7	10.2 $\pm$ 2.7

^aReported is the mean NK-mediated lysis at an effector to target ratio of 10:1, plus or minus standard deviation.

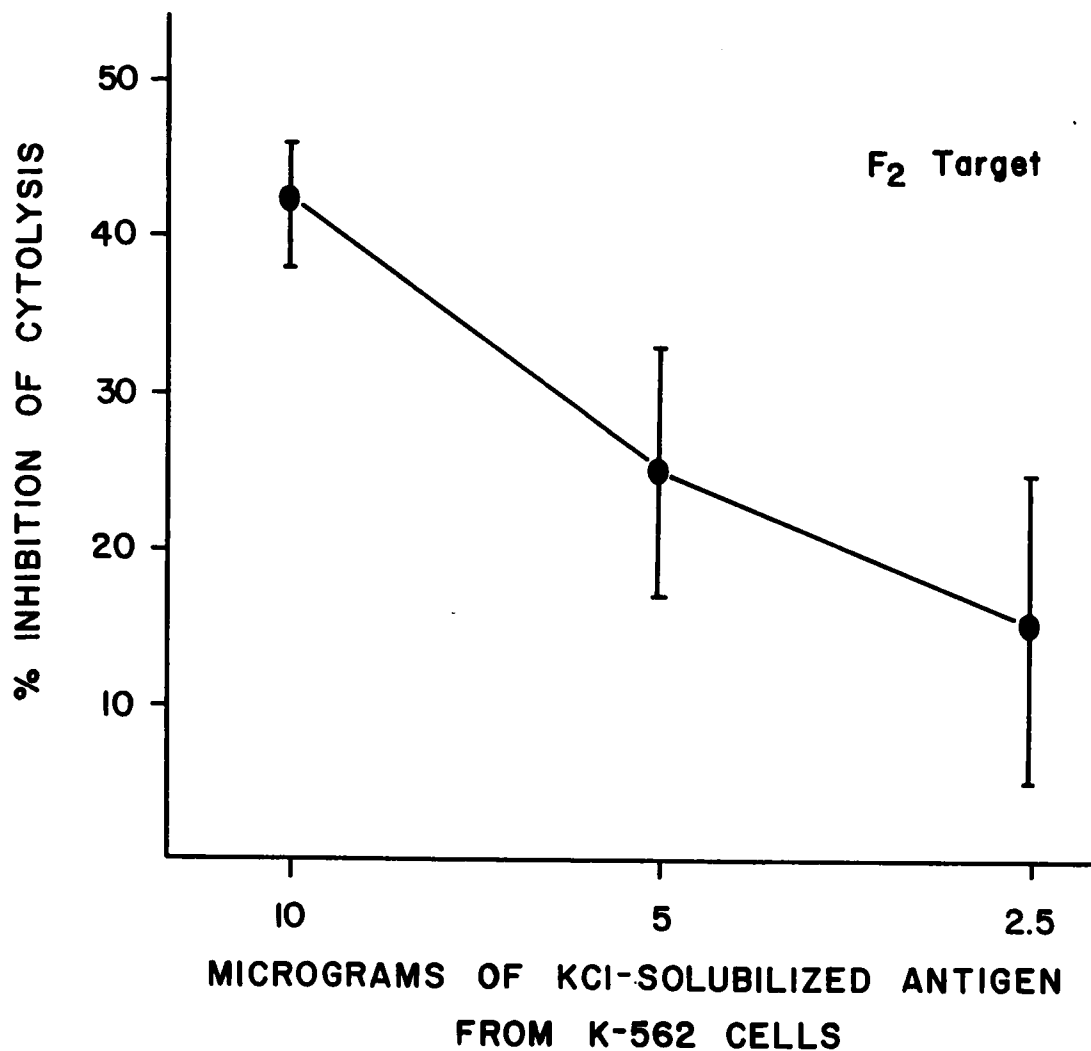


Figure 12. Inhibition of NK-mediated cytolysis by 3M KCl extracts of K-562 cells. Effector to target ratio is 40:1. Maximum lysis with no inhibitor is 45%.

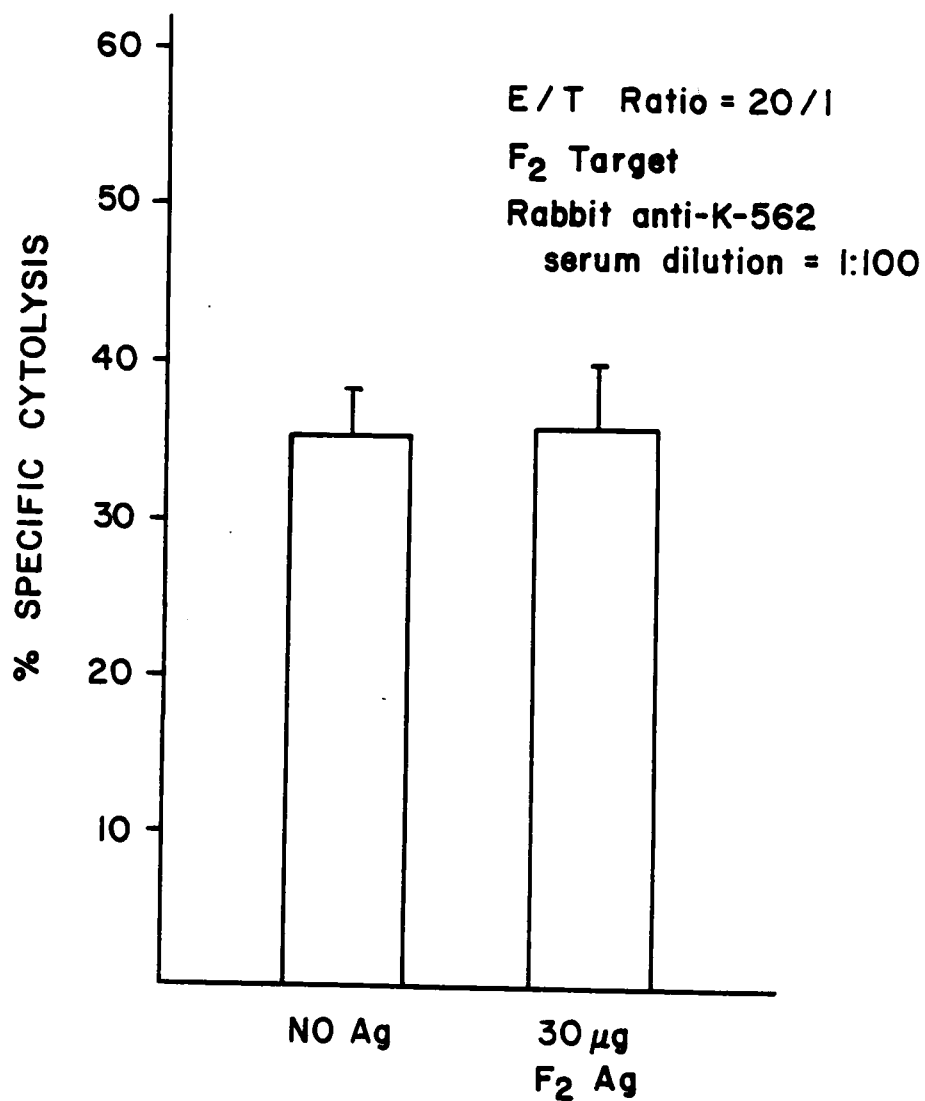


Figure 13. Antibody-dependent cellular killing of K-562 cells in the presence of soluble K-562 extracts.

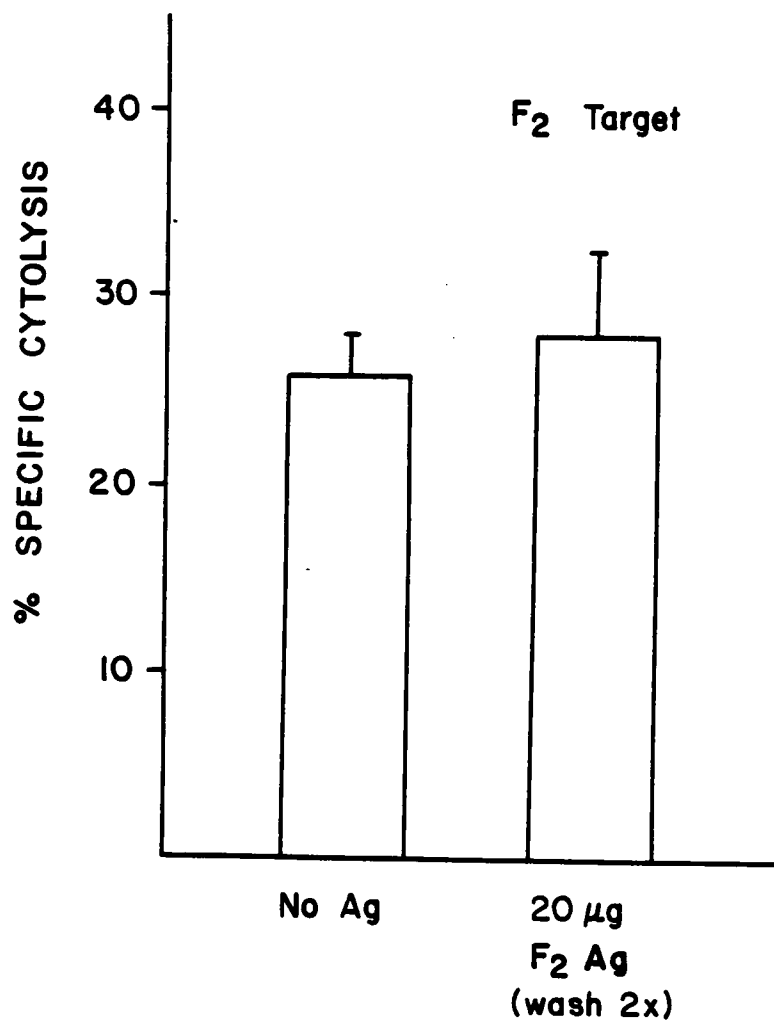


Figure 14. NK-mediated lysis of K-562 cells after preincubation of HPBL with extracted antigen. HPBL were incubated with antigen for 1 hour at 37°C, washed, and assayed for lysis.

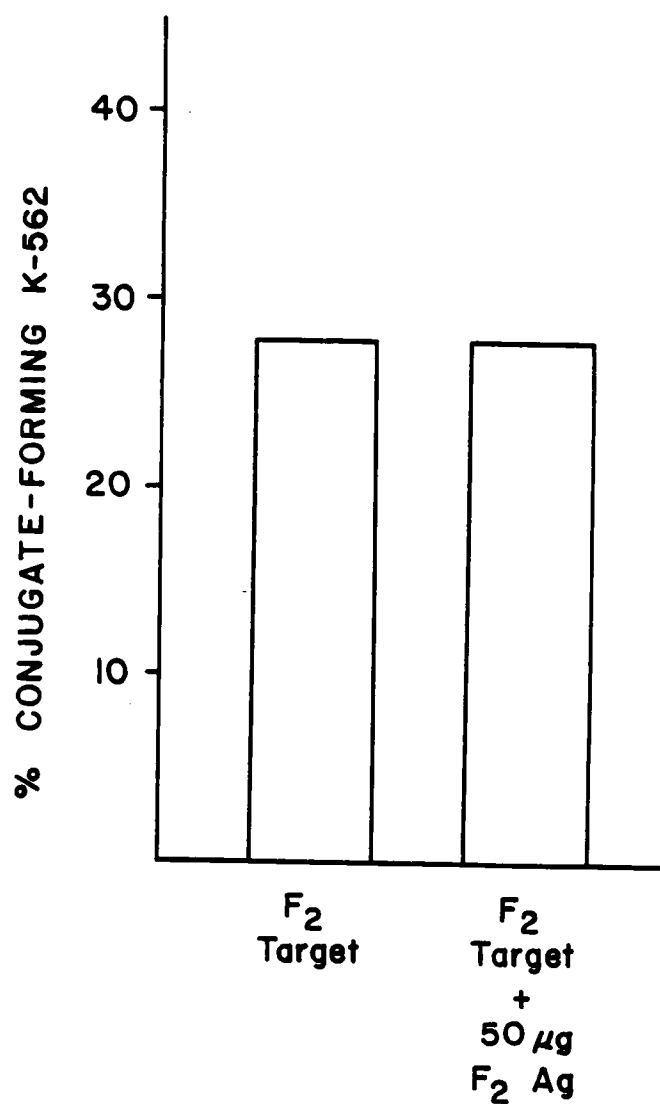


Figure 15. Effector/target conjugate formation by human NK cells in the presence of soluble K-562 antigen.

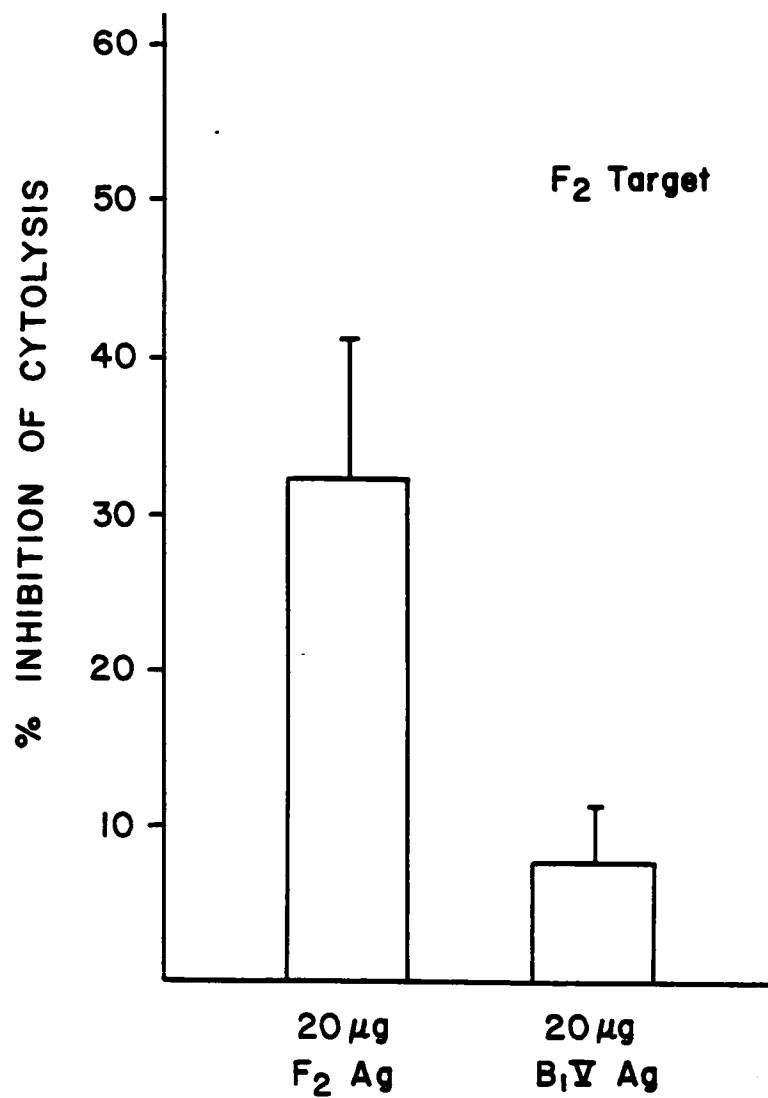


Figure 16. Inhibition of NK-mediated lysis by 3M KCl extracts of K-562 sublines B₁V and F₂.

CHAPTER V

DISCUSSION

An appropriate target cell for NK-mediated cytotoxicity must meet three general criteria:

1. it must express structures that are recognized by the NK cell, allowing for the binding of the NK cell to the target cell;
2. the target cell must provide an activation signal to the NK cell;
3. the target cell must be susceptible to the lytic mechanism of the activated NK cell.

Specificity of lysis caused by NK appears to reside in any of these three criteria. In the first, structures recognized by the NK cell are thought to be protein or glycoprotein in nature (35, 66). Expression of these structures appears to be dependent upon the differentiative state of the target cell (82, 83).

With respect to the second criterion, activation of the NK cell probably involves an interaction of the T-200 glycoprotein complex of the NK cell with some entity on the target cell (74). A monoclonal antibody to a specific determinant on the T-200 complex, will block the cytolytic process at a time subsequent to binding. Since the monoclonal antibody blocks lysis of some targets, such as the K-562 cell, but not others, it has been proposed that specific structures must be present on the target cell (14).

In the third criterion, sensitivity to the NK cell lytic mechanism requires the expression of a specific protease-insensitive receptor for soluble natural killer cytotoxic factors, which are postulated to be involved in lysis (13, 88), as well as susceptibility to their toxic effects. Target cells have been described which appear to have a mechanism that counteracts the lytic action of NKCF (85, 91).

If the target structures recognized by NK cells are truly related to a particular state of differentiation, one might expect a variety of normal cell types to express these structures. Indeed, some subpopulations of normal bone marrow and thymic cells are lysed by NK cells (24). Owing to this recognition of "self", the complex specificity observed in NK-mediated cytotoxicity is likely quite important in defining their range of reactivity.

As to the precise identity of the target structures that denote an NK-sensitive phenotype, little is known. The receptor for transferrin, which is associated with activated cells, has been proposed as constituting one target structure for NK cells (81) but the precise role that it plays is unclear. No other functional membrane proteins have been described as target structures.

In an attempt to identify relevant target structures, a number of investigators have isolated NK-resistant variants of typically NK-sensitive tumor target cells (43, 52, 62), but these have not yielded definitive information with respect to the requisite surface determinants (53). Tumor target cells induced to differentiate, often show a concomitant loss of recognition by NK cells (82, 83). Again the target structures that change are undefined.

in the present study, eight K-562 cell sublines were examined for their susceptibility to lysis by NK cells. These sublines were established from cells that had been frozen at various intervals during passage of the original K-562 cell line. Marked variation was noted amongst these sublines in karyotype and cell surface phenotype (50, A. T. Ichiki, manuscript in preparation).

Considerable variability was found in the susceptibility of these K-562 sublines to lysis by NK cells. A strong correlation was noted between the degree of lysis and modal chromosome number in that predominantly hypodiploid K-562 cell sublines are less efficiently lysed than hypotriploid sublines.

K-562 cells exhibit chromosomal clonal evolution in culture, acquiring abnormal chromosomal characteristics (C. B. Lozzio, personal communication). These changes in karyotype apparently represent a selection for faster growing subtypes. Whether the relative resistance of hypodiploid K-562 sublines to NK activity is directly attributable to karyotypic features is not clear at this time. It is possible that in the transition from diploid to near-triploid, characteristics develop that may favor NK-sensitivity.

It could be that the chromosomal changes noted with passage of K-562 cells are reflective of tumor evolution in the more general sense. It would seem that the susceptibility of K-562 cells to NK is related to this process, the cells acquiring the NK-sensitive phenotype as they evolve in vitro. Possibly the cells originally isolated from the leukemic patient had been selected for resistance to NK in vivo, sensitivity arising after passage of the cells in vitro under

conditions where no selection is taking place. The situation is somewhat analogous to that of the HL-60 cell line in which the uncultured leukemic blasts are resistant to NK although the cultured cell line is sensitive (19).

Considerable heterogeneity exists within any K-562 subline with respect to karyotypic features. Sublines described as significantly hypodiploid often include a considerable proportion of hypotriploid cells (C. B. Lozzio, personal communication). The cloned subline, B₁V, on the other hand, represents a homogeneous population in which ninety-three percent of the cells are hypodiploid (C. B. Lozzio, personal communication).

This cloned subline is resistant to lysis by NK cells. The resistance does not appear to be simply a result of altered kinetics of lysis as has been described with "slow" NK targets (9, 38) because lysis of B₁V occurs rapidly, that is, within the first two hours of incubation. Lysis of sensitive K-562 cells (e.g. subline F₂) is progressive over a 16 hour time period. The kinetics of B₁V lysis might suggest that only a minor population in the culture is sensitive, while the majority of the cells are resistant to lysis. However, the near homogeneity of B₁V may tend to rule out this possibility. Interferon activation of HPBL, which accelerates the kinetics of NK-mediated cytotoxicity (77), had no effect on the relative degree of lysis of B₁V.

B₁V is not resistant to other forms of immune cytotoxicity. Lysis of this line by ADCMC demonstrates that the cells are susceptible to lysis through pore formation by the membrane attack complex of

complement. Recent studies have indicated that lysis by NK cells may be mediated by pore-forming units transferred to the target cell membrane (54). Whether this mode of lysis is comparable to that of complement is not clear, nor is it clear if these lytic factors are actually involved in lysis by NK. The results at least suggest that B₁V is not resistant to NK on the basis of some intrinsic ability to repair lethal membrane damage.

It is possible that B₁V may resist lysis by avoiding the binding or lytic activity of NKCF. These soluble proteins have been identified as the lytic moiety in NK-mediated cytotoxicity (13, 17, 35, 88). The comparable lysis of B₁V and NK-sensitive K-562 by ADCC suggests that B₁V is susceptible to NKCF since these factors have been shown to be responsible for lysis by Fc-positive K cells involved in ADCC (B. Bonavida, personal communication).

NK-resistance in sodium butyrate-induced K-562 cells has been related to a lack of target cell recognition and binding to the NK cell (82, 83), presumably due to a loss or masking of target structures (82, 84). A cloned K-562 line, selected for resistance to NK, has been shown to be resistant at the level of effector cell binding as well (46). Resistance in this cloned line was possibly related to decreased expression of transferrin receptors (43).

B₁V was unable to efficiently compete with NK-sensitive K-562 in NK assays. This might be interpreted to indicate that there is poor binding of NK cells to B₁V. However, conjugate formation assays showed no difference in the NK-binding capacity of B₁V and NK-sensitive K-562 (F₂). Trypsin treatment of the target cells

reduced the degree of binding, presumably by removing target cell recognition structures. Another NK-resistant K-562 subline, Fl, was found to bind relatively few NK cells without such treatment with trypsin. Thus, while the conjugate formation assay appears capable of detecting differences in binding capacity, B₁V was found to bind NK cells at a frequency equal to that of an NK-sensitive K-562 subline. It appears that B₁V cells are recognized normally by NK cells, yet avoid lysis by failing to activate the bound effector cells.

Cold target competitive ability is classically considered as assessing the capacity of a target cell to be recognized by immune effectors (27); however, the poor competitive ability of B₁V, compared with its normal conjugate-forming capacity, could represent a contradiction. This apparent discrepancy may actually provide additional support for the interpretation that B₁V is deficient in triggering the NK lytic process.

Treatment of NK cells with a monoclonal antibody, 13.1, relative with the T-200 glycoprotein complex, leaves the cells unable to trigger for lysis of particular targets, including the K-562 cell (74). In that study it was shown that after treatment of effector cells with 13.1, K-562 cells are no longer able to compete for lysis of Molt-4 lymphoma cells although they still bound effectively to NK cells. Apparently the treated NK cell is not activated upon binding to the K-562 competitor and is still available for lysis of Molt-4 cells. Thus it appears that effector cell activation is required for cold target inhibition by a particular target. This is supported by studies with the NK-insensitive Raji lymphoma line and interferon-treated human

fetal fibroblast targets, both of which effectively bind to NK cells yet compete poorly in cold target inhibition assays (4, 42, 80).

The proposed inefficiency of NK cell triggering by B₁V is also supported by the demonstration of a poor oxidative burst by HPBL in response to B₁V; the resistant line being unable to trigger this early metabolic event in the NK cell lytic process as measured by luminol-dependent chemiluminescence. B₁V is also unable to induce the generation of measurable NKCF.

Presumably the failure of B₁V to activate the lytic mechanism of the NK cell is due to an altered target determinant that is involved in activation subsequent to the binding of the effector cell. Target specificity at the trigger stage has been demonstrated (74) with monoclonal 13.1, described above, which blocks NK cell activation against K-562 but not Molt-4 targets. These findings suggest that specific target determinants are involved in the activation of the effector. If B₁V is truly deficient in delivering this activation signal, it represents a model for study of the target cell-associated "second signal" for NK lysis.

Binding of NK cells to K-562 and subsequent triggering for lysis are both enhanced upon treatment of the target cells with neuraminidase (7). In other studies, neuraminidase-sensitive surface sialic acid has been implicated in masking the recognition structures on sodium-butyrate induced K-562 cells, which lose NK-sensitivity (84). However, neuraminidase treatment of subline B₁V does not increase its susceptibility to lysis by NK cells. The lysis of NK-sensitive K-562

was increased two-fold. Thus, excess surface sialic acid is apparently not responsible for the resistance of B₁V.

It is possible that B₁V lacks a surface protein which is involved in the activation event. It was found that high salt extracts of NK-sensitive K-562 cells inhibited NK-mediated lysis whereas extracts of B₁V did so poorly. This observation supports the interpretation that B₁ lacks a necessary protein determinant. Polyacrylamide gel analysis of these extracts showed no detectable qualitative differences (data not shown). Perhaps the putative trigger structure represents a minor constituent of the cell surface and is thus undetectable on conventional polyacrylamide gels.

Inhibition of NK by high salt tumor extracts appears to be specific. The demonstration of normal lysis by ADCC in the presence of the extracts eliminates mechanisms of inhibition such as overt toxicity to the effector cell. Target cell binding is unaffected by the extracts; thus, they may inhibit by preventing completion of the lytic cycle. HPBL pretreated with extracts of NK-sensitive K-562 and washed, exhibit normal cytolytic activity indicating that the NK cells are not irreversibly inactivated by the extracts.

It is possible that proteins in the soluble extracts of sensitive K-562 may give inappropriate signals at the surface of the NK cell, preventing the cell from mounting the proper response directed at the target. Since intact B₁V cells cannot initiate the lytic mechanism it is reasonable to assume that no inhibitory determinants would be extractable from this line. This is indeed the observed result.

With respect to detectable changes in the cell surface phenotype of B₁V, the only feature of note is a loss of the antigen recognized by the monoclonal anti-Leu antibody (New England Nuclear). This marker is found on all other K-562 lines tested (A. T. Ichiki, personal communication). The nature of this antigen is ill-defined, so the significance of this finding is not clear especially as to its relationship with NK resistance.

B₁V cells express typical levels of transferrin receptor as measured by binding of the monoclonal antibody OKT9, when compared to other K-562 sublines (A. T. Ichiki, personal communication). The cells also express high levels of Fc receptor as measured by rosette formation using antibody coated sheep erythrocytes (data not shown).

In summary, significant variation was seen amongst sublines of K-562 with respect to susceptibility to lysis by human NK cells. Resistance to lysis appeared to correlate with a hypodiploid modal chromosome number. A cloned hypodiploid K-562 subline was resistant to NK-mediated lysis by virtue of failing to bring about the activation of bound NK cells. This defect in effector cell triggering is possibly related to the absence, on the resistant subline, of a surface protein determinant. This proposed determinant would be necessary for the post-binding activation of the NK cell lytic process.

These findings could lead to additional information with respect to the regulation of NK function in vivo. Many cells in the body may express at some time, appropriate differentiation-associated structures for recognition by NK cells. A second-signal requirement may function as an additional regulatory modulator for NK cells. Only appropriate

undifferentiated tumor or hematologic targets expressing both recognition structures and a determinant activating the NK cell lytic mechanism would be subject to lysis.

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